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Inter-sectional hybrids obtained from reciprocal crosses between *Begonia semperflorens* (section *Begonia*) and *B.* 'Orange Rubra' (section *Gaerdita* × section *Pritzelia*)

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Inter-sectional hybrids were successfully obtained by the reciprocal crosses between 11 cultivars (including 6 diploids and 5 tetraploids) of *Begonia semperflorens* (SS & SSSS genomes) and *B.* 'Orange Rubra' (RR genome) with the aid of *in vitro* culture of mature or immature seeds on MS medium containing 0.1 mg l^{-1} α-naphthylacetic acid, 0.1 mg l^{-1} 6-benzyladenine, 10 mg l^{-1} gibberellic acid, 30 g l^{-1} sucrose and 2.5 g l^{-1} gellan gum. Embryo rescue as ovary culture with immature seeds 12^{th}–16^{th} day after pollination (DAP) generally gave higher efficiency of plantlet formation, but in some cross combinations, culture of mature seeds (30 DAP) resulted in higher yield of plantlets. Flow cytometric analysis revealed that they were consisted of the plants with various genomic combinations (RS, RR, RSS, RRS, RRSS and RRRRSS) as estimated by the DNA contents of both parents. Hybridity of these plants with various genomic combinations including RR was confirmed by random amplified polymorphic DNA analysis. These results suggested that unreduced gamete formation and spontaneous chromosome doubling were involved in the hybrid formation of various ploidy levels and genomic combinations. These hybrids showed various levels of intermediate traits between both parents according to the genomic compositions, and some of them had desirable characters of both parents.

Key Words: amphipolyploid, *Begonia* 'Orange Rubra', *Begonia semperflorens*, embryo rescue, inter-sectional hybrid, unreduced gamete.

Introduction

The genus *Begonia* contains about 1400 species (Smith *et al.* 1986). Most of begonias were found naturally throughout the region of tropical/subtropical Africa, Asia and America, but not in Australia (Tebbitt 2005). The greatest number of *Begonia* species occurs in the humid montane forests of South America and mainland Asia (Tebbitt 2005). Owing to the rich colors and beautiful morphologies of flower, striking foliage, free-growth and free-flowering habit, *Begonia* has ensured a place among favorite garden flowers for a long time (Chittend 1951). Especially, some kinds of begonia groups such as tuberous, Rieger, rex and semperflorens begonias have been used frequently in horticultural business for pot plant, annual bedding crop, hanging basket and indoor foliage plant.

Begonia semperflorens is now one of the most important flower crops in the world. It was the fourth largest bedding plant in the United States in 2009, with a total output value of 36 million US dollars (USDA 2010). Compared to other species of *Begonia*, *B. semperflorens* more or less adaptable to different environmental conditions (Bailey 1919). Due to its robust adaptability to the environment and constant blooming all year round, *B. semperflorens* has become an important horticultural crop today. It has been bred for more than one hundred years through interspecific crosses by utilizing several species such as *B. cucullata* (Carriere 1881), *B. schmidtiana* (Zeilinga 1962) and *B. versaliensis* (Hvoslef-Eide and Munster 2006). *B. semperflorens* is the first flower crop, in which F_1 cultivars and polyploid cultivars have been utilized for commercial seed production. Thus, it is often cited as an important example for horticultural evolution (Horn 2002). Although flower colors in the present commercial cultivars of *B. semperflorens* are confined to red, pink and white series, they lack yellow and orange colors. Therefore, it is necessary to hybridize *B. semperflorens* with its distant relatives possessing above expected flower colors for increasing its variations and horticultural utilization.

Interspecific hybridization has utilized frequently to introduce the fine features of a genotype into another genotype so as to improve the original traits of donor materials (Horn 2002) and has successfully created various new plant characteristics in flowering crops, such as *Petunia* (Shaw 2007), *Coleus* (Nguyen *et al.* 2008), carnation (Nimura *et al.* 2003, Umiel *et al.* 1987), poinsettia (Ecke *et al.* 2004), *Euphorbia milli* (Rauh 1979) and most mass-production flower crops. For improving the variation of flower color, interspecific hybridization has successfully been conducted

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to create novel hybrids owing orange flower color in *Impatiens* (Stephens *et al.* 1988) and *Ornithogalum* (Griesbach *et al.* 1993). Besides, the hybrid of *Camellia* displaying a novel golden flower color was produced by the interspecific hybridization between *Camellia chrysantha* (yellow flower color) and *C. japonica* cv. 'Shiratama' × 'Chochidori' (Hwang *et al.* 1992).

Among the genus *Begonia*, there are many species with yellow or orange flower colors. For example, the wild species such as *B. pearcei*, *B. sutherlandii*, *B. cinnabarina* and *B. boliviensis* in the tuberous group and the commercial cultivars of the cane-like group such as *B.* 'Orange Rubra', *B.* 'Eikow', *B.* 'Riviera Sunset' and *B.* 'Mandarine Orange' are the important parental sources to create yellow/orange flower colors (Tebbitt 2005). Compared to the tuberous begonias, those belonging to the cane-like group have better ability to adapt environmental predicament. Moreover, they can well adapt to higher temperature than *B. semperflorens* and tuberous begonia. The huge inflorescence, various flower/leaf colors, silver leaf variegation, and flower fragrance are also the key features to make the cane-like group begonia popular. However, the long stem internode and poor branching habit result in the large/loose plant type and has hindered the cane-like group begonia from wide circulation in the horticultural market. To improve the disadvantages of cane-like group begonia, *B. semperflorens* may be utilized as a parental source for its good features, such as dwarfness and good branching. It is also hoped to create new type of hybrids in *B. semperflorens*, which possess the special characters of cane-like group, such as silver leaf variegation, orange flower colors and flower fragrances.

In the present study, we report the successful results of inter-sectional hybridization by the reciprocal crosses between 11 cultivars of *B. semperflorens* and a cane type cultivar, *B.* 'Orange Rubra' with the aid of embryo rescue technique. *B.* 'Orange Rubra' was inter-sectional hybrids between *B. dichroa* (section *Gaerdita*) and *B.* 'Coral Rubra', which was the progeny between *B. coccinea* (section *Pritzelia*) and unknown male parent (Tebbitt 2005). Furthermore, we report the various ploidy levels in the hybrids due to unreduced gamete formation in both parents. The results on the characterization of these inter-sectional hybrids were also described.

Materials and Methods

Plant materials

Totally twenty-seven cultivars of *Begonia semperflorens* and one plant of *B.* 'Orange Rubra' were used in this study (Tables 1, 2). Among those materials, twenty-three single flower type cultivars of *B. semperflorens* were kindly provided by Sakata Seed Co.; and three double flower type cultivars, one single flower type cultivar 'CU1' of *B. semperflorens* and *B.* 'Orange Rubra' were purchased from a local market. The ploidy level of those *B. semperflorens* cultivars is shown in Table 1. All the plant materials were maintained as potted plants in the greenhouse, which was kept under natural light conditions, whereas the temperature was kept higher than 18°C but below 30°C.

Pollination

The cross of *B.* 'Orange Rubra' × *B. semperflorens* was conducted in the winter of 2008–2009 using *B. semperflorens* 'CU1' as pollen donor. In the reverse cross, a total of 26 cultivars of *B. semperflorens* were used as female parents and the pollination works were conducted during the winter of 2009–2010. During the pollination period, all the male flowers were removed just before anthesis and cultivated at the isolated place to prevent pollination by the other plants. The pollen parent plants were cultivated at the other place to avoid accidental pollination. Fresh pollen was harvested from the male flowers at the first blooming day, then directly pollinated by touching the stigma of maternal parent at the first blooming day.

In vitro seed sowing and embryo rescue

For *in vitro* culture of mature seeds, ovaries were collected 30 days after pollination (DAP), when the fruits initiated to turn brown but before dehiscence. In the embryo rescue experiment, immature ovaries were collected at different DAP period (4, 8, 12, 16 and 20 days). Mature/immature ovaries were surface-sterilized with 70% ethanol for 1 min and then with 1% sodium hypochlorite solution for 15 min containing two drop of Tween 20, followed by 3 times of rinsing with sterile distilled water. After surface sterilization, the placental tissues were excised from ovaries and rolled on the medium carefully. In the case of mature ovaries (30 DAP), all the seeds were released from placental tissue onto the medium, and it was removed thereafter. In younger ovaries (4, 8, 12, 16 and 20 days), however, the placental tissue was kept on medium for the culture of attaching seeds/ovules after the rolling treatment with the released seeds/ovules, which tended to be lesser in younger ovaries. Irrespective of the difference in releasing treatment, they were cultured on embryo rescue (ER) medium, which consisted of full strength MS medium (Murashige and Skoog 1962), 30 g l^{-1} sucrose, 0.1 mg l^{-1} α-naphthylacetic acid (NAA), 0.1 mg l^{-1} 6-benzyladenine (BA), 10 mg l^{-1} gibberellic acid (GA_3) and 2.5 g l^{-1} gellan gum. Besides, another medium suitable for callus induction (PC medium) was used to evaluate their influence on embryo rescue efficiency, in which basic components were the same as above medium, but added with 1 mg l^{-1} thidiazuron (TDZ), 1 mg l^{-1} 4-amino-3,5,6-trichloropyridine-2-carboxylic acid (picloram) instead of NAA and BA. Calluses obtained from embryo rescue cultures were sub-cultured on shoot regeneration medium, which was the full strength MS medium containing 30 g l^{-1} sucrose and 2.5 g l^{-1} gellan gum, supplemented with 0.1 mg l^{-1} NAA and 0.5 mg l^{-1} BA. Whereas shoots regenerated from calli or directly obtained from immature/mature seeds were transferred onto the same basal medium but supplemented with 0.1 mg l^{-1} NAA for root induction. The pH

of media was adjusted to 5.8 with KOH before autoclaving. All cultures were maintained at 24 ± 2°C with 16 hr photoperiod and the light was provided by cool white fluorescent tubes (35 μmol m^{-2} s^{-1}). The progenies were acclimatized and transferred to the greenhouse to investigate the plant morphological traits during 2009–2011.

Measurement of relative nuclear DNA content by using flow cytometry

The relative nuclear DNA contents between parents and progenies were determined by using flow cytometry (Partec GmbH, Munster, Germany). Young leaf segments (about 0.5 cm × 0.5 cm) were chopped with razor blade in 200 μl Solution A (Plant High Resolution DNA kit type P, Partec GmbH), then added with 1 ml DAPI solution (10 mM Tris-HCl, pH 7.5, containing 50 mM sodium citrate, 2 mM $MgCl_2$, 1% (w/v), PVP K-30 (Polyvinylpyrrolidone K-30), 0.1% (v/v) Triton X-100, 2 mg l^{-1} DAPI (4′,6-diamidino-2-phenylindole dihydrochloride) and kept for 1 min to stain the nuclei. The well-mixed solution was filtered through 45 μm nylon mesh to remove the cell debris and analyzed. After preliminary confirmation of chromosome numbers in several cultivars of *B. semperflorens* 'Sprint-Pink' was selected as a diploid cultivar and used as an intrernal standard for flow cytometric analysis of the parental plants and inter-specific hybrids. Peak position for nuclear DNA content of 'Sprint-Pink' was adjusted to channel number 50, relative DNA contents of other cultivars of *B. semperflorens* and *B*. 'Orange Rubra' were determined as relative values provided that nuclear DNA content of 'Sprint-Pink' was 2 units.

DNA extraction and RAPD analysis

Because of the difficulty to isolate DNA from begonia leaves due to the organic acids involved, the protocol of Kopperud and Einset (1995) was used in the present study. About 2 g of mature leaf tissues were ground into a fine powder by using liquid nitrogen, transferred into a centrifuge tube, added with 25 ml of T10E10 washing solution (10 mM tris-HCL, 10 mM EDTA, PH 8.0), vortexed for 10 sec and centrifuged for 10 minutes at 8000 rpm at 4°C. After discarding the supernatant, DNA was extracted by CTAB method (Murray and Thompson 1980).

Among the arbitrary oligonucleotide 10-mer primers of Operon Technologies tested, OPE-03 and OPE-04 were chosen for random *amplified* polymorphic DNA (RAPD) analysis with polymerase chain reaction (PCR) using EX *Taq* polymerase (Takara Co., Japan), in which each 20-μl reaction mixture containing 1 ng total plant DNA was subjected to analysis according to the manufacture's protocol. DNA fragments were amplified with 39 repeating thermal cycles (4 min at 94°C, 30 sec at 94°C, 30 sec at 35°C and 1 min 72°C) in a PTC-200 Peltier thermal cycler (MJ Research, Watertown, MA, USA). After completing PCR, the amplified DNA mixture was loaded on a 2% agarose gel in TAE buffer, and run for 50 min at 100 V. The gel was photographed under UV light to compare the specific RAPD patterns between hybrids and their parents.

Morphological characters and pollen fertility

Various morphological characteristics such as plant height, leaf shape and branching habit were examined to compare the differences between two parents and putative hybrids. Moreover, the other desirable traits such as flower color, leaf color, variegation of silver spots on leaves and flower fragrance were also evaluated to compare the difference. Pollen fertility was assessed by staining with 2% (w/v) aceto-carmine on at least 200 pollen grains in each observation for every genotype with 5 replications.

Result

Fruit set and cross compatibility of B. semperflorens × B. 'Orange Rubra'

When 26 cultivars in five series of *B. semperflorens* were used for inter-sectional crosses of *B. semperflorens* × *B.* 'Orange Rubra', the fruit set ratio of single flower type cultivars including triploids ranged from 70–100% (94.3% in average), which was higher than that of double flower type cultivars (Queen series), which gave 60–70% fruit set ration with the average of 63.3% (Table 1).

Despite of the high fruit set ratios, most of the harvested seeds at maturity 30 DAP were crimpy and failed to germinate, whereas remaining plumpy seeds germinated 2 weeks after inoculation on ER medium. Among the 26 cultivars of *B. semperflorens* used as female parent, 10 cultivars yielded progenies with the plantlet yield efficiency (PYE) of 0.11 to 1.70, whereas 'Ambassador' series containing 8 triploid and 1 tetraploid cultivars was unable to obtain any progenies. In terms of single flower type cultivars, 'Sprint-Pink' gave the highest PYE of 1.70, followed by 'Varsity-White' and 'Monza-White'. On the other hand, PYE of a double flower type cultivar 'Queen-Pink' (1.14) showed almost the same as single flower type cultivars. Unfortunately, the other 2 double flower type cultivars did not yield any progenies.

In embryo rescue experiment (Table 2), four out of five cultivars of *B. semperflorens* examined yielded progenies, whereas 'Ambassador-Coral' (4x) failed to yield plantlets. In the successful cultivars, the highest PYE was obtained when cultured at 12–16 DAP, whereas cultures with earlier or later DAP resulted in reduced PYE (Table 1). Furthermore, the best hybrid efficiencies of 12–16 DAP were obviously higher than 30 DAP in the same cultivar (Tables 1, 2). For instance, the highest PYE of embryo rescue method in *B.* 'Sprint-Pink' × *B.* 'Orange Rubra' was 9.7, which was evidently better than the value (1.7) in the culture of mature seeds.

In both mature and immature seed cultured on ER medium, zygotic embryos germinated as abnormal embryoids without apparent cotyledons after 15 days of culture (Fig. 1A) and only root elongation was observed till 30 days of culture. Then, shoots were formed at the top of abnormal embryo 45 days after culture (Fig. 1B). Although those

Table 1. The efficiency of different cross combinations between *B. semperflorens* and *B.* 'Orange Rubra'

Maternal donor (genome)	No. of flowers pollinated	No. of ovaries cultured[a]	No. of ovaries yielding plantlets[b]	No. of plants obtained	Plantlet yield efficiency[c]
'Monza-Pink' (SS)	10	10	3	6	0.60
'Monza-White' (SS)	10	10	1	7	0.70
'Monza-Coral' (SSSS)	10	10	1	3	0.30
'Monza-Rose' (SSS)	10	10	0	–	–
'Monza-Scarlet' (SSS)	10	10	0	–	–
'Monza-Salmon orange' (SS)	10	9	2	6	0.66
'Monza-Apple blossom' (SS)	10	10	1	4	0.40
'Varsity-Pink imp' (SSSS)	10	9	1	1	0.11
'Varsity-White' (SSSS)	10	10	2	7	0.70
'Varsity-Bicolor' (SSSS)	10	8	1	3	0.37
'Varsity-Rose' (SSS)	10	7	0	–	–
'Ambassador-Coral' (SSSS)	10	8	0	–	–
'Ambassador-Pink' (SSS)	10	10	0	–	–
'Ambassador-White' (SSS)	10	10	0	–	–
'Ambassador-White pink' (SSS)	10	7	0	–	–
'Ambassador-Deep rose' (SSS)	10	10	0	–	–
'Ambassador-Scarlet' (SSS)	10	10	0	–	–
'Ambassador-Rose' (SSS)	10	9	0	–	–
'Ambassador-Rose flash' (SSS)	10	10	0	–	–
'Sprint-Pink' (SS)	10	10	6	17	1.70
'Sprint-White' (SSS)	10	10	0	–	–
'Sprint-Rose' (SSS)	10	10	0	–	–
'Sprint-Red' (SSS)	10	10	0	–	–
'Queen-Pink' (SS)[d]	10	7	3	8	1.14
'Queen-White' (SS)[d]	10	6	0	–	–
'Queen-Red' (SS)[d]	10	6	0	–	–

[a] All the capsules obtained were used for the culture.
[b] Each ovary was inoculated on plate individually.
[c] No. of plants obtained/No. of ovaries cultured.
[d] Double flower cultivars.

rosette shoots did not grow well without development of healthy root system, they developed into normal plantlets after excision and transfer onto root induction medium (Fig. 1D). They were then transferred to pots in soil, acclimatized for at least 3 weeks under sunshade conditions, and successfully grown in greenhouse (Fig. 1E) till flowering to compare the difference of plant characteristics (Fig. 1F).

The effect of different culture medium on the embryo rescue

For evaluating the influence of different culture media on the embryo rescue, the immature fruits were harvested from the cross of *B.* 'Sprint-Pink' × *B.* 'Orange Rubra' at 12 DAP. The result indicated that PC medium had an ability to obtain slightly more progenies than ER medium (Table 3). However, all the progenies on PC medium emerged as calluses and later needed to transfer onto shoot induction medium for regenerating shoots (Fig. 1C). Consequently, PC medium required more time and labor for obtaining normal *in vitro* plantlets than ER medium.

Estimation of hybridity, ploidy and genomic composition by using flow cytometry

Totally 142 progenies, which were obtained from 9 cross combinations between *B. semperflorens* and *B.* 'Orange Rubra', were subjected to flow cytometry (FCM) analysis to evaluate the relative DNA contents of both parental donors and their hybrids (Table 4 and Fig. 2). The relative DNA contents of *B. semperflorens* diploid cultivars, tetraploid cultivars and *B.* 'Orange Rubra' were 2, 4 and 4.74 units, respectively. In the case of cross combination between *B. semperflorens* diploid cultivar (SS genome) and *B.* 'Orange Rubra' (RR genome), more than half (59%, 72/122) of the hybrids showed expected normal genome combination (RS) with relative DNA content of 3.37 unit (Fig. 2A). Some unexpected DNA contents that might correspond to the different genomic combinations of both parents such as RRS (Fig. 2B), RSS, RRSS (Fig. 2C) and RRRRSS were also obtained as 4.37, 5.74, 6.78 and 11.48 units, respectively. A noteworthy fact is that the RRRRSS genome appeared only in the cross combination between diploid *B. semperflorens* 'Monza-Pink' and *B.* 'Orange Rubra' (Fig. 2D).

In the 3 cross combinations between tetraploid cultivars of *B. semperflorens* (SSSS genome) and *B.* 'Orange Rubra' (RR genome), 83% progenies (25/30) showed the expected triploid genome combination (RSS) with relative DNA

Table 2. The effect of different DAP on the embryo rescue between *B. semperflorens* and *B.* 'Orange Rubra'

Maternal donor	Days after pollination	No. of ovaries cultured	No. of ovaries yielding plantlet[a]	No. of plantlet obtained	Plantlet yield efficiency[b]
'Monza-Pink' (SS)	4	3	1	4	1.33
	8	3	1	3	1
	12	3	3	15	5
	16	3	3	21	7
	20	3	3	14	4.66
'Varsity-Pink imp' (SSSS)	4	3	0	–	–
	8	3	1	1	0.33
	12	3	3	6	2
	16	3	3	11	3.66
	20	3	3	1	0.33
'Ambassador-Coral' (SSSS)	4	3	0	–	–
	8	3	0	–	–
	12	3	0	–	–
	16	3	0	–	–
	20	3	0	–	–
'Sprint-Pink' (SS)	4	3	3	14	4.66
	8	3	3	16	2.66
	12	3	3	29	9.66
	16	3	3	20	6.66
	20	3	3	17	5.66
'Queen-Pink' (SS)	4	3	1	2	0.66
	8	3	0	–	–
	12	3	3	5	1.66
	16	3	1	7	2.33
	20	3	1	1	0.33

[a] Each ovary was inoculated on a plate individually.
[b] No. of plants obtained/No. of ovaries sown on the medium.

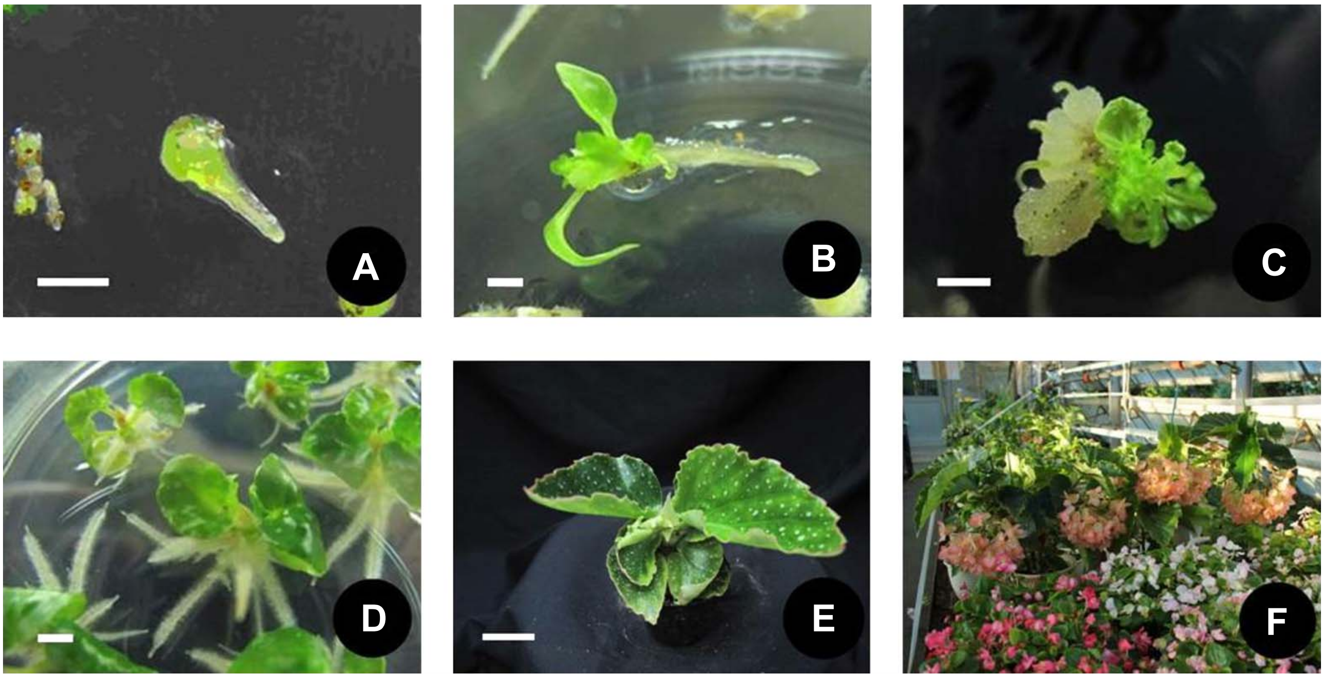

Fig. 1. Regeneration of hybrid plants after reciprocal crosses between *B. semperflorens* and *B.* 'Orange Rubra'. (A) Germinated embryos from immature seeds 15 days after inoculation onto ER medium. (B) Shoot regeneration from the top of abnormal embryo 45 days after culture on ER medium. (C) Shoot regeneration from callus, which was obtained from immature seed 30 days after culture on PC medium, after transfer onto shoot induction medium. (D) Hybrid plantlets established after transfer onto root induction medium. (E) Hybrid plant between *B. semperflorens* 'Queen-Pink' and *B.* 'Orange Rubra' established in pot 6 months after acclimatization. (F) Flowering of the hybrid in greenhouse about 1.5 years after initiation of embryo rescue culture. Bar = 0.2 cm for (A)~(D) and 2 cm for (E).

Table 3. The influence of different culture medium on the embryo rescue efficiency in the inter-sectional hybridization between *B. semperflorens* 'Sprint-Pink' and *B.* 'Orange Rubra'

Medium[a]	No. of ovaries inoculated[b]	No. of progenies obtained	Efficiency of cross combination[c]	Growth response
PC medium	5	39	7.8	Callus
ER medium	5	21	4.2	Embryoids & Shoot

[a] PC medium: MS medium supplement with 1 mg l^{-1} TDZ, 1 mg l^{-1} picloram, and 10 mg l^{-1} GA_3.
ER medium: MS medium supplement with 1 mg l^{-1} NAA, 0.1 mg l^{-1} BA and 10 mg l^{-1} GA_3.
[b] Each ovary was harvested on 12 DAP and inoculated on a plate individually.
[c] No. of plants obtained/No. of ovaries inoculated on the medium.

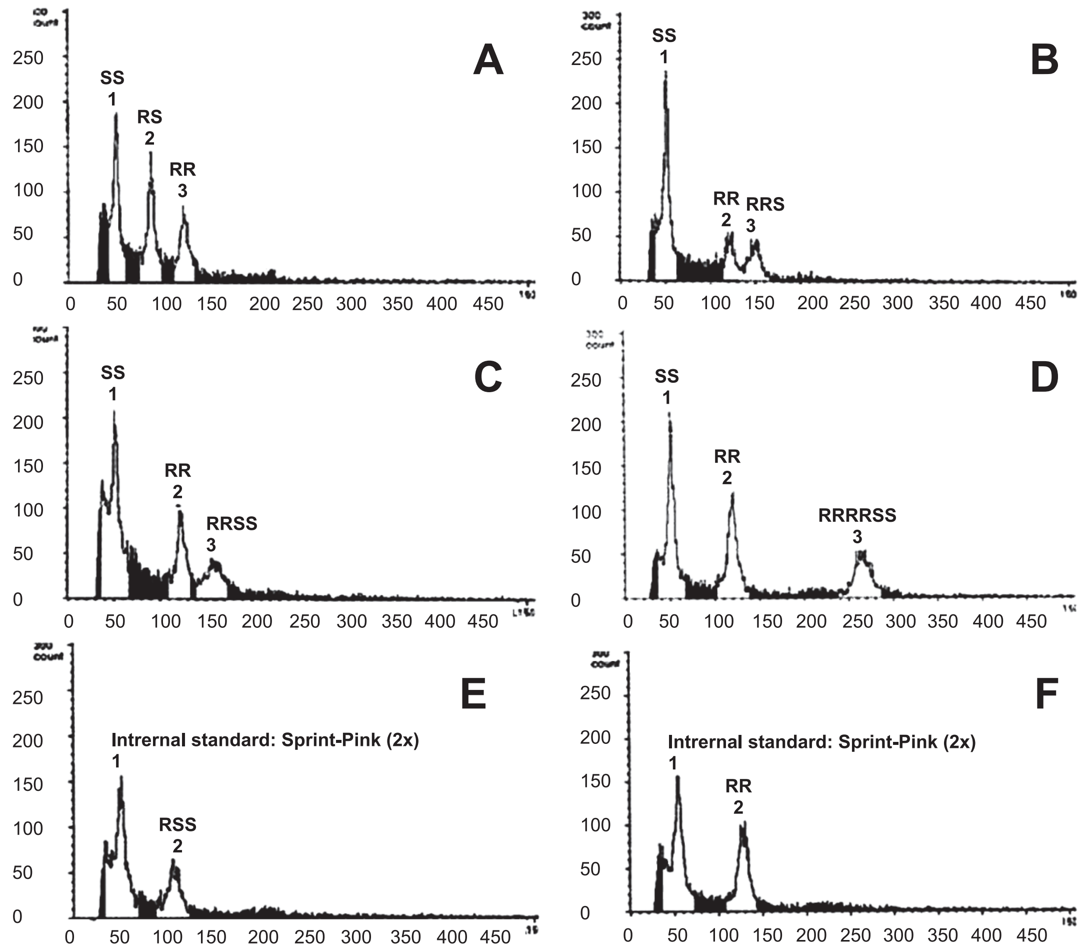

Fig. 2. Relative DNA content of hybrids between *B. semperflorens* and *B.* 'Orange Rubra' were estimated by flow cytometric analysis. (A) 1: *B. semperflorens* 'Queen-Pink' (SS). 2: Hybrid (RS). 3: *B.* 'Orange Rubra' (RR). (B) 1: *B. semperflorens* 'Monza-Pink' (SS). 2: *B.* 'Orange Rubra' (RR). 3: Hybrid (RRS). (C) 1: *B. semperflorens* 'Monza-Apple blossom' (SS). 2: *B.* 'Orange Rubra' (RR). 3: Chromosome-doubled Hybrid (RRSS). (D) 1: *B. semperflorens* 'Monza-Pink' (SS). 2: *B.* 'Orange Rubra' (RR). 3: Chromosome-doubled hybrid (RRRRSS) presumably produced after fertilization of 'Monza-Pink' with unreduced pollen of 'Orange Rubra'. (E) 1: *B. semperflorens* 'Sprint-Pink' (2x). 2: Hybrid (RSS) obtained from *B.* 'Orange Rubra' × *B. semperflorens* 'CU1' (SSSS). (F) 1: *B. semperflorens*, 'Sprint-Pink' (2x). 2: Hybrid (RR) obtained from *B.* 'Orange Rubra' × *B. semperflorens* 'CU1' (SSSS).

content of ca. 4.37 unit. However, the plants with unexpected genome combinations, RRS and RRSS, were also produced in the crosses using *B.* 'Monza-Coral' (4x) in the former and both 'Monza-Coral' and 'Varsity-White' in the latter, respectively.

In addition, the relative DNA content of the hybrids obtained from reverse cross combination, *B.* 'Orange Rubra' (RR) × tetraploid *B. semperflorens* 'CU1' (SSSS), was also investigated (Table 5). Among 12 progenies totally analyzed, only 2 were revealed to be expected RSS hybrids (Fig. 2E) with 4.37 units, whereas remaining 10 progenies (83%) displayed completely equal DNA content to that of *B.* 'Orange Rubra' (4.74 units, RR genome) (Fig. 2F).

Table 4. Flow cytometric analysis of the ploidy level and genome combination of hybrids in inter-sectional cross between *B. semperflorens* and *B.* 'Orange Rubra'

Cross combination / Maternal donor	No. of hybrids obtained[a]	No. of hybrids analyzed	Pollen donor: *B.* 'Orange Rubra' RR = 4.74 units					
	Expected genome combination		RS	RSS	RRS	RRSS	RRSSSS	RRRRSS
	Relative DNA Content (units)		3.37	4.37	5.74	6.74	8.74	11.48
Diploid cultivars of *B. semperflorens* (2x) SS = 2 units								
'Monza-Pink'	56	27	12	1	8	1		5
'Monza-White'	7	2	2					
'Monza-Salmon orange'	6	6	3	3				
'Monza-Apple blossom'	4	4	3			1		
'Sprint-Pink'	113	62	52	3	7			
'Queen-Pink'	23	11		7	4			
	Expected genome combination			RSS	RRS	RRSS	RRSSSS	RRRRSS
	Relative DNA Content (units)			4.37	5.74	6.74	8.74	11.48
Tetraploid cultivars of *B. semperflorens* (4x) SSSS = 4units								
'Monza-Coral'	3	3		1	1	1		
'Varsity-Pink imp'	20	20		20				
'Varsity-White'	7	7		4		3		

[a] The hybrids were obtained from mature seed sowing method and embryo rescue method in cases where diploid and tetraploid *B. semperflorens* were used for the crosses, respectively.

Table 5. Flow cytometric analysis of the ploidy level and genome combination of hybrids in inter-sectional cross between *B.* 'Orange Rubra' and *B. semperflorens* 'CU1'

Cross combination / Maternal donor	No. of hybrids analyzed[a]	Pollen donor: Tetraploid cultivars of *B. semperflorens* 'CU1' (4x) SSSS = 4 units	
	Expected genome combination	RSS	RR
	Relative DNA Content (units)	4.37	4.74
B. 'Orange Rubra' RR=4.74 units	12	2	10

[a] Relative nuclear DNA content estimated through flow cytometric analysis of DAPI-stained nuclei.

Hybridity analysis by using RAPD markers

DNA bands specific to *B. semperflorens* cultivars and *B.* 'Orange Rubra' were detected by RAPD analysis using the primers OPE-4 and OPE-3, among the 10 random primers tested. The putative hybrids 2R, 6R 19R, 29R and QR, obtained from the different cross combinations between *B. semperflorens* and *B.* 'Orange Rubra', showed the specific bands for both parents using OPE-4 (Fig. 3A). Although the putative hybrids 23R and 28R did not show the specific band of *B. semperflorens* by OPE-4 primer, the hybridity was confirmed by another primer OPA-2 (data not shown). Moreover, hybridity of the putative hybrids obtained from the reverse crosses, i.e., *B.* 'Orange Rubra' × *B. semperflorens* 'CU1' was also confirmed by using the primer OPE-3 even in the plants that showed RR genome type (Fig. 3B). Thus, all the progenies obtained from both reciprocal crosses were confirmed to be hybrids.

Characteristics of the hybrids

In the crosses where *B. semperflorens* was used as female parents, two triploid hybrids with different genomic types (RRS and RSS) already attained to flowering stage (Fig. 4A, 4B). Both types of hybrids showed almost intermediate plant height but differed in flower color depending on the genomic combinations; RRS plant had more Orange Rubra-like flowers whereas RSS showed more *B. semperflorens*-like ones (Fig. 4A, 4B). In the reverse crosses where tetraploid *B. semperflorens* was used as male parent, hybrid plants with RSS genome showed almost the same dwarf plant height as *B. semperflorens* (Fig. 4C). Although RR genome type plants were also obtained in this cross combination, they also showed the same dwarf phenotype as RSS hybrids (Fig. 4D). In these two genome types of hybrids, RSS plants bore almost the same size of flowers to *B. semperflorens*, whereas RR plants produced comparable size of flowers to *B.* 'Orange Rubra'. One of the interesting results found in the hybrid phenotypes was the leaf variegation recognized as silver spots on leaves (Fig. 4E). Although the character of silver spots is originally found in *B.* 'Orange Rubra' to some extent but not in any cultivars of *B. semperflorens*, it was expressed more strongly in the hybrids than *B.* 'Orange Rubra' (Fig. 4E). Moreover, we also observed the flower fragrance in triploid (RRS) hybrid (Fig. 4A), which had stronger expression than the other hybrids and *B.* 'Orange Rubra'.

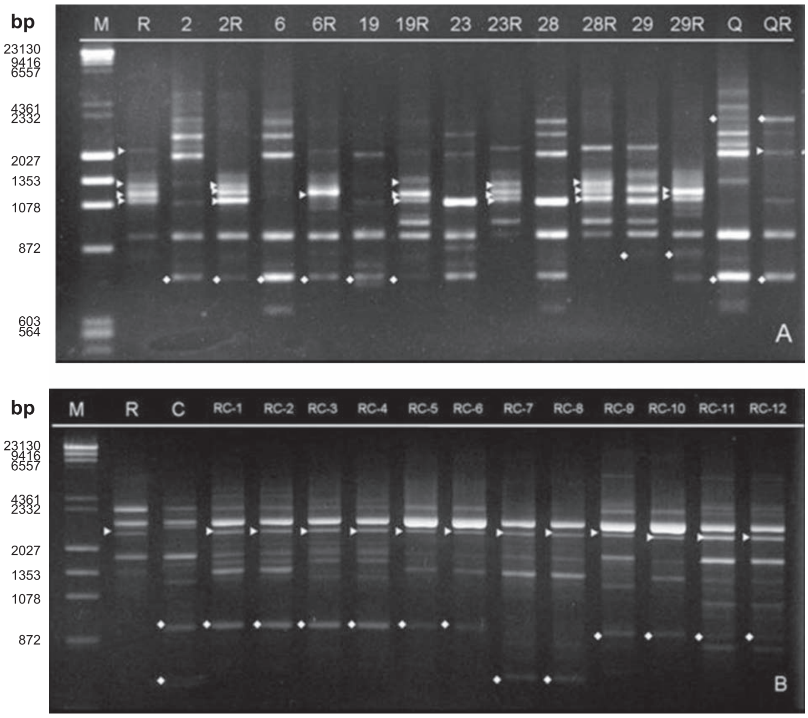

Fig. 3. RAPD analysis for confirming the hybridity of the palnts obtained from the crosses between *B. semperflorens* and *B.* 'Orange Rubra' (A) and from the reverse cross (B). (A) The primer OPE-04 was used. R: 'Orange Rubra'. 2: 'Monza-Pink'. 6: 'Varsity-White'. 19: 'Sprint-Pink'. 23: 'Varsity-Pink imp'. 28: 'Monza-Apple blossom'. 29: 'Monza-Salmon orange'. Q: 'Queen-Pink'. The hybrid of 2R, 6R, 19R, 23R, 28R, 29R and QR were obtained from different cross combinations between each cultivar (2, 6, 19, 23, 26 and 29) of *B. semperflorens* and *B.* 'Orange Rubra'. (B) The primer OPE-03 was used. R: *B.* 'Orange Rubra'. C: *B. semperflorens* 'CU1'. RC1-RC12: All the 12 hybrids between 'Orange Rubra' and 'CU1' containing both RSS and unexpected RR genome plants. M: λDNA/*Hin*dIII + Φ174/*Hae*III-digestion size marker. ▷ The specific band of *B.* 'Orange Rubra'. ◇ The specific band of *B. semperflorens*.

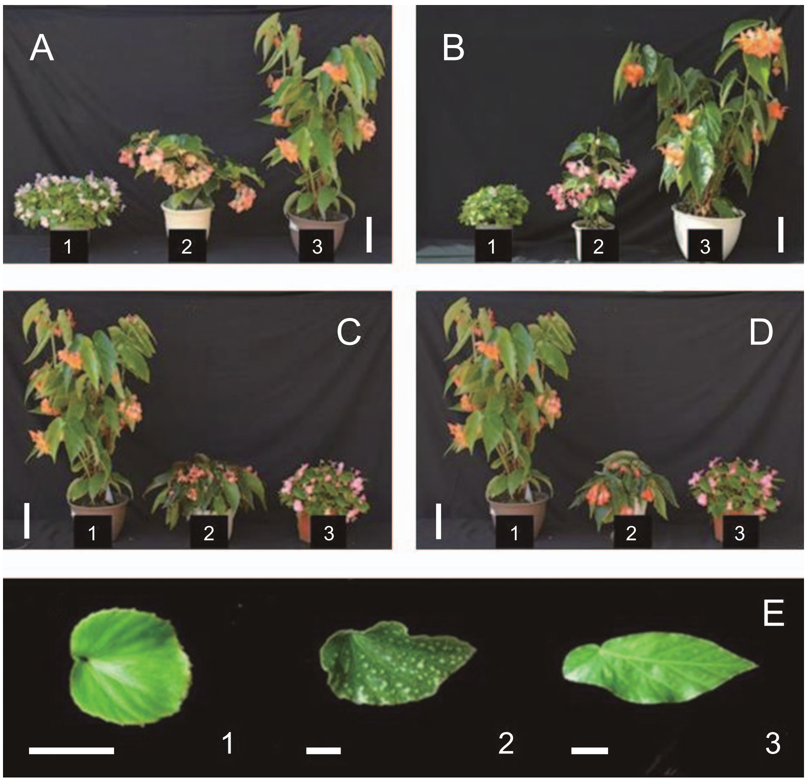

Fig. 4. Characteristics of hybrid plants obtained from reciprocal crosses between *B. semperflorens* and *B.* 'Orange Rubra'. (A) 1: Female parent: *B. semperflorens* 'Sprint-Pink'. 2: Hybrid 'SO1' (RRS). 3: Male parent: *B.* 'Orange Rubra'. (B) 1: Female parent: *B. semperflorens* 'Varisity-Pink imp'. 2: Hybrid 'VO1' (RSS). 3: Male parent: *B.* 'Orange Rubra'. (C) & (D) 1: Female parent: *B.* 'Orange Rubra'. 2 (C): Hybrid 'OC1' (RSS); 2 (D): Hybrid 'OC3' (RR). 3: Male parent: *B. semperflorens* 'CU1'. (E) Comparison of leaves among 1: Female parent: *B. semperflorens* 'Queen-Pink'. 2: Hybrid (RS) between *B. semperflorens* 'Queen-Pink' and *B.* 'Orange Rubra'. 3: Male parent: *B.* 'Orange Rubra', Bar = 15 cm for (A)~(D) and 1.5 cm for (E).

Pollen fertility of hybrids

The pollen fertilities of parental plants examined were as follows: 72.2% in 'Sprint-Pink' (2x), 20.4% in 'Varsity-Pink imp' (4x), 39.2% in 'CU1' and 74.1% in 'Orange Rubra', respectively (Table 6), whereas triploid cultivars of *B. semperflorens* had almost no pollen fertility. Among the hybrids which attained to the flowering stage, all the triploid hybrids including RRS genome (SO1–SO3) obtained from *B. semperflorens* 'Sprint-Pink' × *B.* 'Orange Rubra' and RSS genome (VO1–VO5 and OC1–OC2) obtained from *B. semperflorens* 'Varsity-Pink imp' × *B.* 'Orange Rubra' and reverse cross, *B.* 'Orange Rubra' × *B. semperflorens*

Table 6. Pollen fertility of *B. semperflorens*, *B.* 'Orange Rubra' and their hybrids with different genomic compositions[a]

Genotype	Ploidy	Genome	Pollen fertility (%)[b]
Female parent			
B. semperflorens 'Sprint-Pink'	2x	SS	72.2 b
B. semperflorens 'Varsity-Pink imp'	4x	SSSS	20.4 g
Hybrids of *B. semperflorens* × *B.* 'Orange Rubra'			
B. 'Sprint-Pink' × *B*. 'O. R.' (SO1) (Fig. 4A)		RRS	0 h
B. 'Sprint-Pink' × *B*. 'O. R.' (SO2)		RRS	0 h
B. 'Sprint-Pink' × *B*. 'O. R.' (SO3)		RRS	0 h
B. 'Varsity- Pink imp' × *B*. 'O. R.' (VO1) (Fig. 4B)		RSS	0 h
B. 'Varsity- Pink imp' × *B*. 'O. R.' (VO2)		RSS	0 h
B. 'Varsity- Pink imp' × *B*. 'O. R.' (VO3)		RSS	0 h
B. 'Varsity- Pink imp' × *B*. 'O. R.' (VO4)		RSS	0 h
B. 'Varsity- Pink imp' × *B*. 'O. R.' (VO5)		RSS	0 h
Male parent/Female parent			
B. 'Orange Rubra'	2x	RR	74.1 a
Hybrids of *B*. 'Orange Rubra' × 4x *B. semperflorens*			
B. 'Orange Rubra' × *B*. 'CU1' (OC1) (Fig. 4C)		RSS	0 h
B. 'Orange Rubra' × *B*. 'CU1' (OC2)		RSS	0 h
B. 'Orange Rubra' × *B*. 'CU1' (OC3) (Fig. 4D)		RR[c]	44.9 c
B. 'Orange Rubra' × *B*. 'CU1' (OC4)		RR[c]	24.1 f
B. 'Orange Rubra' × *B*. 'CU1' (OC5)		RR[c]	41.1 d
Male parent			
B. semperflorens 'CU1'	4x	SSSS	39.2 e
LSD			0.313

[a] Means with different case letters in a column are significantly different by least significant difference (LSD) test at $p < 0.05$.
[b] Pollen with normal morphology and stained with aceto-carmine was counted as fertile pollen.
[c] These RR plants had corresponding DNA content to that of *B.* 'Orange Rubra' but showed evidence of the hybridity with *B. semperflorens* by RAPD analysis (see Fig. 3B).

'CU1' showed almost no pollen fertility. However, the diploid hybrids possessing almost equal amount of DNA to 'Orange Rubra' (RR type) obtained from the cross of *B*. 'Orange Rubra' × *B. semperflorens* 'CU1' showed relatively high pollen fertility (24–45%) which was comparable to *B.* 'Orange Rubra'.

Discussion

In the present study, inter-sectional hybrids have successfully been obtained from the crosses between *B. semperflorens* cultivars and a cultivar of cane-type group, *B*. 'Orange Rubra'. Since the hybrids could be obtained from mature seeds without using embryo rescue technique, sexual barriers between these two parental plants might not be so firm even though they are phylogenetically isolated into different sections. These results suggested that pollination of Orange Rubra's pollen could induce parthenocarpic response in ovules of *B. semperflorens* or that triploid plants had the ability to produce female gametes, which could be at least fertilized. Although hybrids were obtained by *in vitro* culture of mature seeds at low efficiencies, application of immature seed culture technique for rescuing the relatively early stages of embryos, i.e., 12–16 DAP, was confirmed to be beneficial in all the cross combinations tested (Tables 1, 2). In the interspecific hybridization, hybrid embryos are sometimes abnormal and require tissue culture process for obtaining normal plants (Amano *et al.* 2006). In the present study, hybrid embryos were mostly abnormal in morphology and required *in vitro* culture for obtaining normal plant regeneration. For the safe and successful production of interspecific hybrids, therefore, embryo rescue technique should be generally applied especially in the case of the plants, which have minute seeds such as *Begonia*. Although Peng and Chiang (2000) reported the presence of unilateral incompatibility in the reciprocal crosses between *Begonia formosa* and *Begonia aptera*, it was not observed in the present reciprocal crosses between *B. semperflorens* × *B.* 'Orange Rubra'.

In the genus *Primula*, various genomic combinations have been obtained in the interspecific hybrids (Amano *et al.* 2006, Hayashi *et al.* 2007a, 2007b, 2009, Kato and Mii 2000, Kato *et al.* 2001) and the unreduced gamete has been confirmed as the main factor for production of the polyploid interspecific hybrids (Kato *et al.* 2008). In terms of the genus *Begonia*, Dewitte *et al.* (2010) inferred that the meiotic aberration during 2n pollen formation was one of the main reasons for leading to the occurrence of various chromosome numbers in *Begonia*. In the present study, various combinations of parental genomes were found in the inter-sectional hybrids obtained from reciprocal crosses between

B. semperflorens and *B.* 'Orange Rubra' based on the data of FCM analysis (Tables 4, 5). In the crosses of diploid *B. semperflorens* × *B.* 'Orange Rubra', totally 4 unexpected genome types were observed (RSS, RRS, RRSS and RRRRSS) in addition to the normal genome combination (RS). These results suggest that unreduced gamete formation occurred in both female gamete yielding RSS hybrids and male gamete yielding RRS hybrids and that chromosome doubling also occurred in both diploid hybrid yielding RRSS hybrids and triploid hybrid yielding RRRRSS hybrids. In the cross between tetraploid *B. semperflorens* (SSSS) × *B.* 'Orange Rubra' (RR), two unexpected genome combinations (RRS and RRSS) were also found in addition to the expected normal combination (RSS) in the hybrids. The tetraploid hybrids (RRSS) could be originated from the fertilization between normal reduced female gamete (SS) and unreduced male gamete (RR). On the other hand, it is rather difficult to explain how RRS hybrid was induced. Although RR genome might be induced as unreduced male gamete, it is unusual to produce monoploid female gamete (S) in tetraploid plant of *B. semperflorens*. Further analysis on this hybrid might be necessary.

In the reverse cross, i.e., *B.* 'Orange Rubra' × tetraploid *B. semperflorens* 'CU1', most of the progeny plants (10/12) had the relative DNA content corresponding to RR genome combination (4.74 units) except for the few expected normal hybrids with RSS genome (4.37 units) (Table 5). Although these RR plants showed very similar morphology to *B.* Orange Rubra, they had dwarf morphology like as *B. semperflorens* (Fig. 4D) and clear specific bands of *B. semperflorens* in RAPD analysis (Fig. 3B). These results suggest that they were not produced by accidental selfing of *B.* 'Orange Rubra' but that they were induced after fertilization to produce RSS genome, followed by elimination of SS genome and chromosome doubling to produce RR type progeny. It is also possible that the elimination of SS genome occurred after fertilization of unreduced female gamete of *B.* 'Orange Rubra' to produce RRSS genome. During these processes, minor fragments of S genome might have been incorporated into RR genome without causing apparent change in DNA content. Possible occurrence of chromosome translocation and chromosome deletion was also reported previously in the hybrids of Taiwanese *Begonia*, as the reason for the frequent appearance of aneuploid during the natural polyploidization process (Oginuma and Peng 2002).

Advantageous expression of useful characters has been observed in other interspecific hybridizations conducted in various genera such as *Cosmos* (Oku *et al.* 2008), *Primula* (Amano *et al.* 2006), *Allium* (Nomura *et al.* 2002) and *Kalanchoe* (Izumikawa *et al.* 2008). In the present study, we have successfully created inter-sectional hybrids by reciprocal crosses between *B. semperflorens* and *B.* 'Orange Rubra' with several different genomic combinations of each parent. Since some of these hybrids have some novel useful characters such as dwarfness, abundant flowers, novel flower colors, conspicuous silver spots on leaf and strong fragrance, they could be directly utilized as novel cultivars by vegetative propagation even if they are sterile. Moreover, they could be eventually used as the breeding materials to produce novel crops with intermediate characters of both parents or for the introgression of useful characters of *B.* 'Orange Rubra' into *B. semperflorens* or vice versa. Since all triploid hybrids (RRS or RSS) examined had no pollen fertility, it is necessary to restore the fertility by artificial chromosome doubling. Since other hybrids with different genomic constitutions or ploidy levels have not yet attained to flowering stage, those triploid hybrids already flowered seem to have shorter juvenile period than the other genomic combination of hybrids. The results on the detailed characterization of the hybrids with different genomic combinations will be summarized in the near future.

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